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(2)

REPORT DOCUMENTATION PAGE

DTIC FILE COPY

AD-A202 746

1b. RESTRICTIVE MARKINGS	
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
3. DISTRIBUTION / AVAILABILITY OF REPORT	Approved for public release; distribution is unlimited
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	NMRI 88-5
5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION	Naval Medical Research
6b. OFFICE SYMBOL (If applicable)	
7a. NAME OF MONITORING ORGANIZATION	Naval Medical Command
6c. ADDRESS (City, State, and ZIP Code)	Bethesda, Maryland 20814-5055
7b. ADDRESS (City, State, and ZIP Code)	Department of the Navy Washington, D.C. 20372-5120
8a. NAME OF FUNDING / SPONSORING ORGANIZATION	Naval Medical Research and Development Command
8b. OFFICE SYMBOL (If applicable)	
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code)	Bethesda, Maryland 20814-5055

10 SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
62770A	3M162770A870	AF 312-1	DA301614

11. TITLE (Include Security Classification)
Allelic forms of gp195, a major blood-stage antigen of Plasmodium Falciparum, are expressed in liver stages

12. PERSONAL AUTHOR(S) Szarfman A, Walliker D, McBride JS, Lyon JA, Quakyi IA, Carter R

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM ____ TO ____	1988	6

16. SUPPLEMENTARY NOTATION
Reprinted from: Journal of Experimental Medicine January 1988 Vol. 167 pp. 231-236

17. COSATI CODES

FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Animal
Antigens, Protozoan
Liver
Malaria
Plasmodium Falciparum

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

DTIC
S ELECTE D
DEC 2 3 1988
H

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Phyllis Blum, Information Services Division	202-295-2188	ISD/ADMIN/NMRI

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

ALLELIC FORMS OF gp195, A MAJOR BLOOD-STAGE ANTIGEN OF *PLASMODIUM FALCIPARUM*, ARE EXPRESSED IN LIVER STAGES

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Current efforts to produce vaccines against the malaria parasite *Plasmodium falciparum* have concentrated on antigens of sporozoites, asexual blood forms, and gametocytes (1). Little attention, however, has been paid to antigens of exoerythrocytic (EE) forms of the parasite which develop in the liver from sporozoites inoculated by mosquitoes. EE forms are less accessible for study than the other stages, and mature parasites are difficult to obtain either in vitro or in vivo. An antigen has been identified that appears to be specific to EE forms (2). Merozoites of EE forms initiate the blood infection, and it is therefore likely that they also possess surface proteins that are structurally and functionally equivalent to those of blood-form merozoites. Previous efforts to demonstrate this have not been successful (3). Once the blood infection is established, the parasite burden increases 10–20-fold every 48 h, making it increasingly difficult to achieve sterile immunity. A vaccine that produces an immune response against both the EE and erythrocytic stage would markedly increase the chance of developing protective immunity. A major glycoprotein of *P. falciparum* blood-form schizonts and merozoites (4), denoted here as gp195, is currently under consideration as a potential vaccine antigen (5–9). This is a polymorphic protein (10–12), which also possesses highly conserved regions. In this study we show that conserved and allele-specific epitopes of gp195 present in *P. falciparum* blood forms are also expressed in mature EE forms. The inheritance of these allele-specific epitopes in a cross between these two parasite clones shows that mature EE forms, like sporozoites and blood stages, are genetically haploid.

This work was supported in part by Naval Medical Research and Development Command Work unit 3M162770A870AF312 (A. Szarfman), the World Health Organization Special Program for Research and Training in Tropical Diseases (I. A. Quakyi), and the Medical Research Council of Great Britain (D. Walliker). The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resource, National Research Council, Department of Health and Human Resources, Publication No. (NIH) 85-23.

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Materials and Methods

Procedure for Obtaining Mature EE Forms and Blood Forms of P. falciparum. Mature EE forms of *P. falciparum* were obtained in the livers of splenectomized chimpanzees (*Pan troglodytes*). Each chimpanzee was inoculated with sporozoites derived from mosquitoes (*Anopheles freeborni*) that had fed on cultured gametocytes, as described previously (13). One animal (CH/3D7) was infected with sporozoites of a *P. falciparum* clone denoted 3D7, a second (CH/HB3) with sporozoites of a clone denoted HB3, and a third (CH/X) with sporozoites derived from a mixture of 3D7 and HB3 gametocytes that had undergone cross-fertilization in the mosquitoes (13). A liver biopsy was taken from each animal 6 d after infection.

Blood forms were detected in each chimpanzee 10 d after sporozoite inoculation, and established in in vitro cultures in human red cells.

Immunofluorescence Assays (IFAs). These tests were performed, at pH 7.3, on liver sections from chimpanzees and on cultured blood-stage shizonts. Liver sections (2 μm) were prepared by cryostat sectioning and examined for parasites by phase-contrast microscopy and Giemsa staining. The chimpanzees had previously been used in studies on hepatitis viruses; therefore, residual viruses were killed by fixing cryostat sections with 1% formalin in PBS for 10 min, followed by three washes for 10 min in PBS. The sections were dried, wrapped in aluminum foil, and stored at −70°C for not more than 4 wk before use. Every fifth cryostat section was stained with Giemsa's stain and examined for EE schizonts by light microscopy. Sections adjacent to these sections were thawed and used for IFAs.

Blood-stage schizonts were prepared for IFA after 12 d of culture. Parasites were prepared on multispot microscope slides, air dried, fixed, and processed in a similar manner as were liver-stage parasites.

The liver schizonts and blood-stage schizonts of CH/3D7 and CH/HB3 were examined for reactivity with a panel of mAbs against gp195. mAb 7B2 (14), (used as positive control) and a pool of appropriate negative controls, 20–50 mAbs of different isotypes against *Trypanosoma* and *Rickettsia* species were interjected at random. Fluorescein-labeled goat anti–mouse Ig (IgA, IgG, and IgM) was obtained from CooperBiomedical, Inc., Malvern, PA. Double-immunofluorescence tests were performed on liver sections and blood-stage schizonts of CH/X. Each preparation was incubated with a mixture of mAbs 7.3 (IgG2a) and 9.2 (IgG1), washed three times in PBS, and then stained with a mixture of two fluorescent reagents: (*a*) a fluorescein-conjugated goat anti–mouse IgG2a, and (*b*) a rhodamine-conjugated goat anti–mouse IgG1 from Southern Biotechnology Associates, Inc., Birmingham, AL. Liver schizonts were identified by phase-contrast and fluorescent microscopy, located by Vernier coordinates, photographed with Kodak Ektachrome 400 film, then stained with Giemsa, and located again for additional photographs. At least 20 liver schizonts were examined for reaction with each mAb. The goat anti–mouse Ig reagents were used at a concentration that avoided false positive reactions.

mAbs against gp195. The mAbs against blood-stage gp195 used in this study were 7.3, 9.2, and 9.8 (10), and 7B11, 7B2, 7H10, 3B10, 7F1, and 4G12 (14). Some of these mAbs recognize different epitopes of gp195 (14) and its merozoite-associated products. Three mAbs, 7.3, 9.2, and 7B11, recognize serotype-restricted epitopes and the other six recognize common epitopes to all isolates tested (10, 14). All mAbs were used at 10–40 μg/ml.

Results and Discussion

EE schizonts, with a maximum diameter of ~95 μm, were detected in the liver of each chimpanzee (Fig. 1). In Giemsa-stained sections, they appeared mature, with small nuclei in a granular cytoplasm. Individual merozoites could be distinguished in most schizonts. The presence of gp195 epitopes on both blood and EE schizonts was shown by IFA with nine mAbs that recognize epitopes representing most regions of the molecule (14). In EE schizonts a dense granular

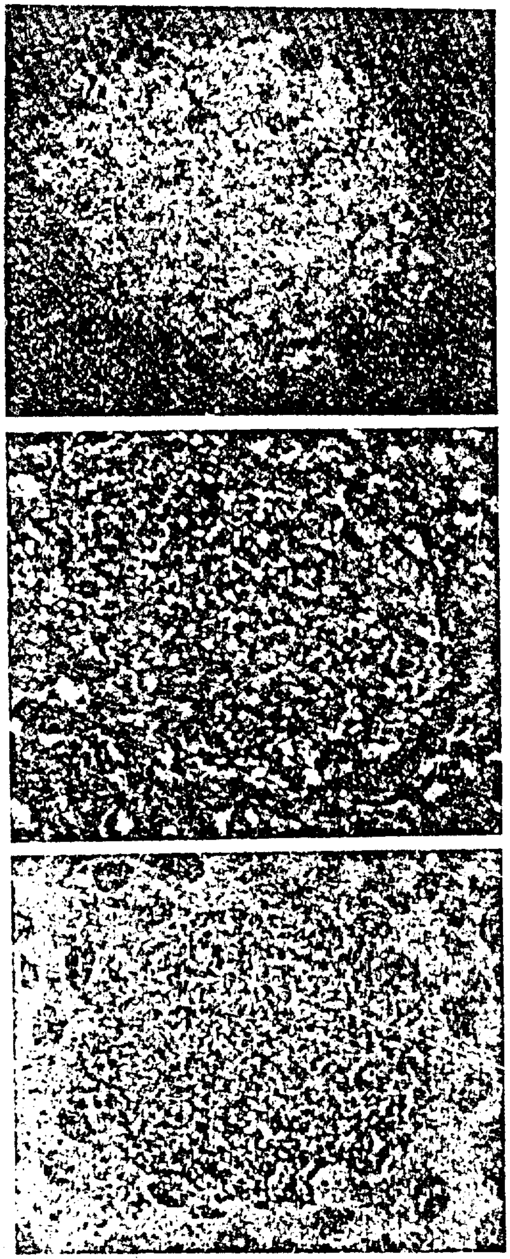

FIGURE 1. (*Top*) Positive immunofluorescence of a liver schizont of *P. falciparum* showing the typical dense granular pattern of gp195. Largest diameter of this schizont was 80 μm; (*middle* and *bottom*) phase microscopy and Giemsa staining of the same parasite.

pattern of staining was seen with each parasite antibody (Fig. 1). In mature blood forms, the staining was particularly evident on the surface of merozoites, as described previously (10). Six mAbs (7B2, 7H10, 3B10, 7F1, 4G12, and 9.8) reacted positively with both liver and blood forms derived from all three chimpanzees. One mAb (7.3) reacted with liver and blood schizonts derived from CH/HB3 but not CH/3D7; two (9.2, 7B11) reacted with CH/3D7 but not CH/HB3. In the third chimpanzee (CH/X), schizonts positive and negative for each of these three mAbs were detected (Table I). The reactivities of each mAb were identical for the liver and blood forms of each respective parasite clone (Table I).

These findings provide evidence that gp195 is present in EE forms as well as

TABLE I

Immunofluorescence Reactions of mAbs Recognizing gp195 of P. falciparum with EE and Blood-Stage Schizonts

mAb	Immunofluorescence reactivity*					
	EE schizonts			Blood-stage schizonts		
	3D7	HB3	X	3D7	HB3	X
7.3	−	+	±	−	+	±
9.2 and 7B11	+	−	±	+	−	±
7B2, 7H10, 3B10, 7F1, 4G12, and 9.8	+	+	+	+	+	+

* Similar signal intensity was observed with EE and blood-stage schizonts. Immunofluorcence reactivity was graded from ++++ to −. When positive reactions were found, mAbs 7.3, 7B2, 7H10, 3B10 were ++++; mAbs 9.2, 7B11, and 9.8 were +++; and mAbs 7F1 and 4G12 were ++. Clones 3D7 and HB3, and parasites derived from a mixture of gametocytes of each clone (X), were used as antigens. In CH/X, ± indicate a mixture of positive and negative schizonts.

TABLE II

Segregation of Allele-specific Epitopes of gp195 among EE Schizonts in Chimpanzees CH/3D7, CH/HB3, and CH/X

EE schizonts	Total examined	Number of schizonts positive for epitopes		
		7.3 only	9.2 only	Both 7.3 and 9.2
CH/3D7	32	0	32	0
CH/HB3	63	63	0	0
CH/X	66	42	24	0

Sections of liver were incubated with mixtures of mAbs 7.3 and 9.2 stained with a mixture of fluorescein-conjugated goat anti-mouse IgG2a (recognizing mAb 7.3) and rhodamine-conjugated goat anti-mouse IgG1 (recognizing mAb 9.2), and examined by IFA.

in blood schizonts of *P. falciparum*. If the binding of these antibodies to parasites had been nonspecific, they would have been expected to react equally with both 3D7 and HB3. The fact that they reacted in a clone-specific manner with both blood forms and EE schizonts provides strong evidence that the primary structure of the antigen is the same in both stages.

The genetics of gp195 in EE schizonts was further investigated with double-immunofluorescence tests. The gp195 antigen exists in the *P. falciparum* blood-stage population as a series of distinct alleles (11–13), those of 3D7 and HB3 being distinguishable by mAbs 7.3, 9.2, and 7B11. Liver schizonts and blood-form schizonts were incubated with mixtures of mAbs 7.3 and 9.2, followed by staining with a fluorescein-labeled antibody specific for mAb 7.3 (IgG2a) and a rhodamine-labeled antibody specific for 9.2 (IgG1). In CH/HB3, the parasites exhibited labeling only with fluorescein, and in CH/3D7 only with rhodamine. In CH/X, schizonts were labeled with either fluorescein or rhodamine; none were labeled with both reagents (Table II).

This result establishes that mature EE forms, like sporozoites (15) and blood forms (16), are genetically haploid. A concurrent study (13) has established that cross-fertilization between 3D7 and HB3 gametes occurred at a very high frequency in the mosquitoes that provided the sporozoites for infection of CH/X. If the resulting EE forms were diploid, it would be expected that a large

proportion of them would exhibit both forms of gp195 that distinguish the parental lines. The absence of such EE forms shows that segregation of the alleles determining the variant forms of this antigen had occurred before the EE stage of the life cycle. Cytological studies using electron microscopy, have shown that synaptonemal complexes, characteristic of meiosis, are present in the zygote stage in the mosquito (17). It can be concluded, therefore, that the entire cycle in the mammalian host is haploid.

The use of cloned parasites that are distinguishable by their reactivity with different mAbs has helped us establish that epitopes of gp195 of mature liver stages of *P. falciparum* are antigenically identical to the ones present in blood schizonts. In previous studies by Druilhe et al. (3), mAbs against unspecified antigens of blood stages gave negative reactions in sections of EE forms of *P. falciparum* obtained in a *Cebus apella* monkey. Here we have shown that multiple epitopes of the gp195 blood-form antigen are also present in mature EE forms of distinct clones of the parasite in chimpanzees. The findings lead us to suggest that other blood-stage antigens may also be shared by EE stages. If immune responses against gp195 and other shared antigens control the development of *P. falciparum*, they could be effective not only against blood-stage parasites, but also against merozoites emerging from the liver.

Summary

Mature exoerythrocytic (EE) forms of two cloned lines (3D7 and HB3) of *Plasmodium falciparum* were obtained in the livers of splenectomized chimpanzees. Sectioned preparations were examined by immunofluorescence (IFA) using mAbs that distinguished allelic variants of the blood-form antigen gp195 and mAbs that recognized multiple conserved epitopes of gp195. EE forms and blood schizonts exhibited identical IFA reactions for each respective clone, showing that the antigen was expressed identically in liver and blood-stage parasites. A third chimpanzee was infected with sporozoites derived from a mixture of 3D7 and HB3 gametocytes that had undergone cross-fertilization in the mosquitoes. IFAs on the EE forms in this animal showed that segregation of each gp195 allele had occurred earlier in the life cycle, providing evidence that the parasite is haploid for the whole of its mammalian development. (Reprints)

We thank Drs. L. H. Miller, F. A. Neva, W. E. Collins, W. T. London, R. Wistar, Jr., and Dr. S. Hoffman for valuable discussions.

Received for publication 29 September 1987 and in revised form 28 October 1987.

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